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pREP4

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User Manual

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Kit Contents and Storage

Shipping and Storage

- pREP4 vectors are shipped on wet ice. Upon receipt, store vectors at –20ºC.
- Store the stab at room temperature.

Contents

- 20 µg of pREP4 is supplied at 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL.
- 10 µg pREP4/CAT is supplied at 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 20 µL.
- 1 stab of TOP10 *E. coli*

Note

We recommend that you prepare a glycerol master stock (page 4) prior to using the TOP10 *E. coli*.

Genotype of TOP10

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac* 74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Introduction

Product Overview

Description of the System

pREP4 is an episomal mammalian expression vector that uses the Rous Sarcoma Virus long terminal repeat (RSV LTR) enhancer/promoter for transcription of recombinant genes inserted into the multiple cloning site. The Epstein-Barr Virus replication origin (oriP) and nuclear antigen (encoded by the EBNA-1 gene) is carried by the plasmid to permit extrachromosomal replication in human, primate and canine cells. pREP4 also carries the hygromycin B resistance gene for stable selection in transfected cells.

pREP4/CAT is provided as a control for the relative level of expression of recombinant proteins in a cell line of interest. pREP4/CAT expresses the chloramphenicol acetyl transferase protein from the RSV LTR enhancer/promoter. pREP4/CAT contains the hygromycin B resistance gene for selection.

Experimental Outline

Use the following outline to clone and express your gene of interest in pREP4.

1. Consult the multiple cloning site depicted on page 3 to design a strategy to clone your gene into pREP4.
2. Ligate your insert into the pREP4 vector and transform into *E. coli*. Select transformants on LB plates containing 50 to 100 µg/mL ampicillin.
3. Analyze transformants for the presence of your insert by restriction digestion.
4. Select a transformant with the correct restriction pattern and use sequencing to confirm that the gene is cloned in the proper orientation.
5. Transfect the construct into a mammalian cell line using your own method of choice. Generate a stable cell line, if desired.
6. Test for expression of the recombinant gene by western blot analysis or functional assay.

Methods

Cloning into pREP4

Before Starting

A diagram is provided on the next page to help you design a cloning strategy for ligating your gene of interest into pREP4. General considerations for cloning and transformation are listed below.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

E. coli Strain

Many *E. coli* strains are suitable for the propagation of this vector, including TOP10 and DH5α. We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A-deficient (*end*A).

For your convenience, TOP10 is available as chemically competent or electrocompetent cells from Invitrogen (see page 11 for ordering information).

Transformation Method

You may use any method of your choice for transformation. Chemical transformation is the most convenient for most researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintaining pREP4

To propagate and maintain pREP4, use the supplied 0.5 µg/µL stock solution in TE, pH 8.0 to transform a *recA*, *endA E. coli* strain and select transformants on LB plates containing 50 to 100 µg/mL ampicillin. Be sure to prepare a glycerol stock of your plasmid-containing E. coli strain for long-term storage (see page 4).

Kozak Sequence for Mammalian Expression

If you will be recombining your entry clone with a destination vector for mammalian expression, your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position –3 and the G at position +4 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

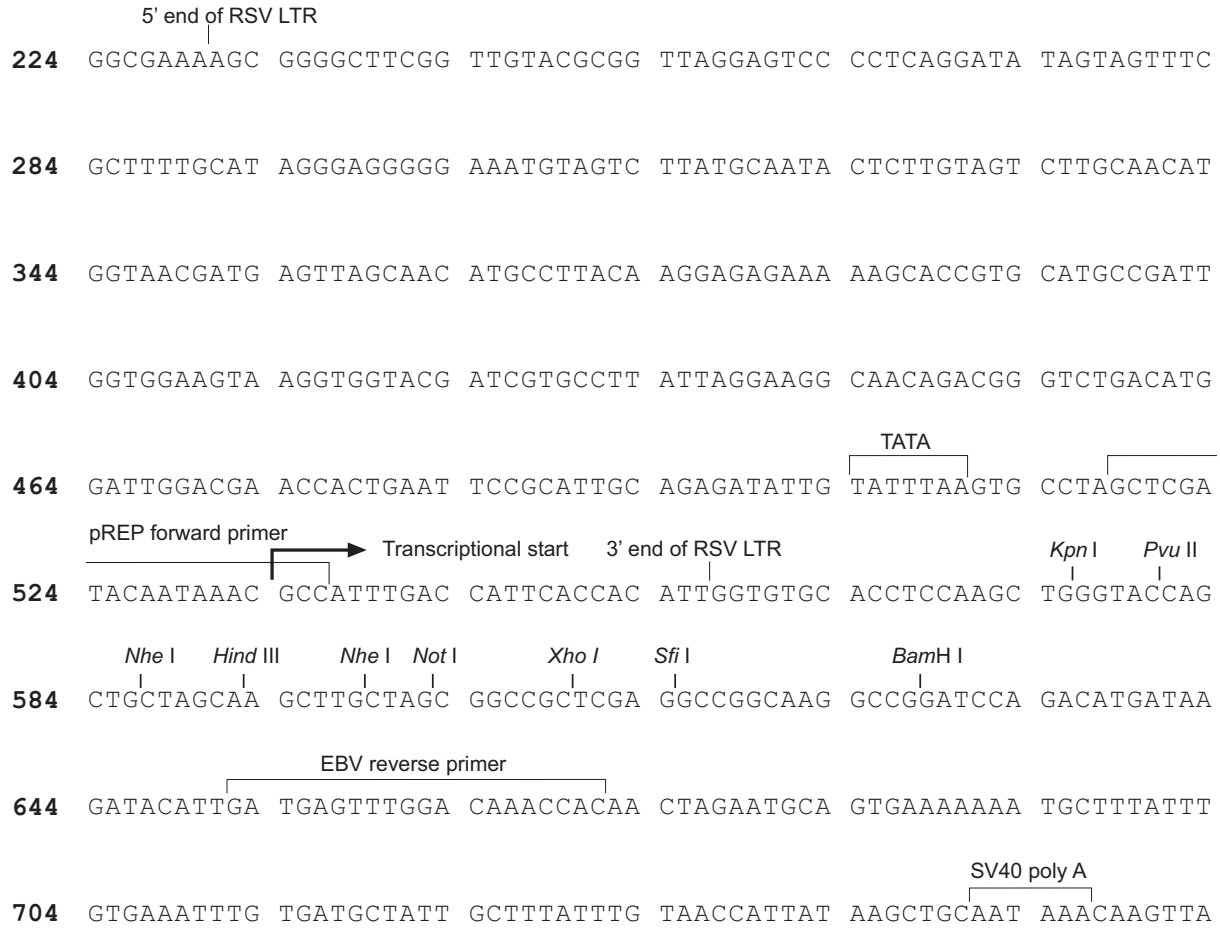
(G/A)NNATG**G**

Continued on next page

Cloning into pREP4, Continued

Multiple Cloning Site of pREP4

Below is the multiple cloning site for pREP4. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. The sequence of pREP4 is available at www.invitrogen.com or from contacting Technical Support (see page 12).


5' end of RSV LTR
224 GGCGAAAAGC GGGGCTTCGG TTGTACGCGG TTAGGAGTCC CCTCAGGATA TAGTAGTTTC
284 GCTTTTGCAT AGGGAGGGGG AAATGTAGTC TTATGCAATA CTCTTGTAGT CTTGCAACAT
344 GGTAACGATG AGTTAGCAAC ATGCCTTACA AGGAGAGAAA AAGCACCGTG CATGCCGATT
404 GGTGGAAGTA AGGTGGTACG ATCGTGCCTT ATTAGGAAGG CAACAGACGG GTCTGACATG
TATA
464 GATTGGACGA ACCACTGAAT TCCGCATTGC AGAGATATTG TATTTAAGTG CCTAGCTCGA
pREP forward primer
Transcriptional start
3' end of RSV LTR
Kpn I
Pvu II
524 TACAATAAAC GCCATTTGAC CATTCACCAC ATTGGTGTGC ACCTCCAAGC TGGGTACCAG
Nhe I
Hind III
Nhe I
Not I
Xho I
Sfi I
BamH I
584 CTGCTAGCAA GCTTGCTAGC GGCCGCTCGA GGCCGGCAAG GCCGGATCCA GACATGATAA
EBV reverse primer
644 GATACATTGA TGAGTTTGGA CAAACCACAA CTAGAATGCA GTGAAAAAAA TGCTTTATTT
SV40 poly A
704 GTGAAATTTG TGATGCTATT GCTTTATTTG TAACCATTAT AAGCTGCAAT AAACAAGTTA

*Note that there are two *Nhe* I sites in the polylinker.

Continued on next page

Cloning into pREP4, Continued

E. coli Transformation

Transform your ligation mixtures into a competent *recA, endA E. coli* strain (e.g., TOP10) and select transformants on LB plates containing 50 to 100 µg/mL ampicillin. Select 10–20 clones and analyze for the presence and orientation of the insert.

Preparing a Glycerol Stock

After identifying the correct clone, purify the colony and make a glycerol stock for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at –20°C.

1. Streak the original colony on an LB plate containing 50 µg/mL ampicillin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50 µg/mL ampicillin.
3. Grow the culture to mid-log phase (OD_{600} = 0.5–0.7).
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Applying Selective Pressure

Take some (if not all) of the following precautions to prevent your clone from being "overrun" by background contaminants:

- **Use carbenicillin instead of ampicillin.** Carbenicillin is more stable than ampicillin, and allows for a longer period of selective pressure, thus preserving your clones longer.
- **Increase the antibiotic concentration.** More antibiotic means that your clones will not be overwhelmed by β-lactamase buildup.
- **Decrease growth times.** Minimize the amount of time that a clone can grow in the media so as to avoid β-lactamase buildup.
- **Periodically refresh plate media.** If you suspect that tubes/plates may be beginning to fail, spin them down, remove the old media, and replenish the wells with fresh LB media plus glycerol and antibiotic.

Streak clones on selective (preferably carbenicillin) LB agar plates. After about 12 hours, isolate colonies for downstream usage. This will isolate your desired clones from potential background contaminants.

Transfection

Introduction

Once you have verified that your gene is cloned in the correct orientation and contains an initiation ATG and a stop codon, you are ready to transfect your cell line of choice. We recommend that you include the positive control vector and a mock transfection (negative control) to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit or the PureLink™ HiPure Plasmid Midiprep Kit, available from Invitrogen (see page 11 for ordering information).

Methods of Transfection

For established cell lines (e.g. HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. Precisely follow the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (**Reference** section, page 14).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). For high efficiency transfection in a broad range of mammalian cells, use Lipofectamine™ 2000 Reagent available from Invitrogen (see page 11). For more information on Lipofectamine™ 2000 and other transfection reagents, visit www.invitrogen.com or contact Technical Support (see page 12).

Positive Control

pREP4/CAT is provided as a positive control vector for mammalian transfection and expression (see page 10) and may be used to optimize transfection conditions for your cell line. The gene encoding chloramphenicol acetyl transferase (CAT) is expressed in mammalian cells under the control of the RSV LTR enhancer/promoter. A successful transfection will result in CAT expression that can be easily assayed (see below).

Assay for CAT Protein

If you want to assay for CAT protein using an ELISA assay, you may use the FAST CAT® Chloramphenicol Acetyltransferase Assay Kit available from Invitrogen (see page 11 for ordering information). Other commercial kits for CAT assay are also available.

Creating Stable Cell Lines

Hygromycin Resistance

pREP4 contains the hygromycin resistance gene for selection of stable cell lines using hygromycin B. Test the sensitivity of your mammalian host cell to hygromycin B, as natural resistance varies among cell lines. General information and guidelines are provided in this section for your convenience.

Hygromycin B Activity

Hygromycin B (527.5 MW) is an aminocyclitol that inhibits protein synthesis by disrupting translocation and promoting mistranslation. Hygromycin B-phosphotransferase detoxifies hygromycin-B by phosphorylation.

- Hygromycin is light sensitive. Store the liquid stock solution at 4°C protected from exposure to light.
- Hygromycin is toxic. Do not ingest solutions containing the drug.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling hygromycin and hygromycin-containing solutions.

Determining Antibiotic Sensitivity

To successfully generate a stable cell line expressing your gene of interest from pREP4, determine the minimum concentration of hygromycin B required to kill your untransfected host cell line. Typically, concentrations ranging from 10 to 400 µg/mL hygromycin are sufficient to kill most untransfected mammalian cell lines. We recommend that you test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your host cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of hygromycin (0, 10, 25, 50, 100, 200, 400 µg/mL hygromycin).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of hygromycin that prevents growth within 2–3 weeks after addition of hygromycin.

Note: Cells will divide once or twice in the presence of lethal doses of hygromycin, so the effects of the drug may take several days to become apparent. Complete inhibition of cell growth can take 2–3 weeks in selective medium.

Continued on next page

Creating Stable Cell Lines, Continued

Selecting Stable Integrants

Once the appropriate hygromycin concentration is determined, generate a stable cell line with your construct.

1. Transfect your mammalian host cell line with your pREP4 construct using the desired protocol. Remember to include a plate of untransfected cells as a negative control and the pREP4/CAT plasmid as a positive control.
2. 24 hours after transfection, wash the cells and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing hygromycin at the pre-determined concentration required for your cell line. Split the cells such that they are no more than 25% confluent.
4. Replenish selective medium every 3–4 days until hygromycin-resistant foci can be identified.
5. Pick and expand colonies in 96- or 48-well plates.

Maintaining Stable Transfectants

Note that pREP4 is an **episomally-maintained** plasmid (Reisman and Sugden, 1986; Yates *et al.*, 1985). Transfected cells may lose the pREP4 plasmid if they are not maintained under selection or are continuously cultured for long periods of time (over six months). To prevent loss of pREP4 from transfected cells, we recommend that you follow these guidelines when working with the cells:

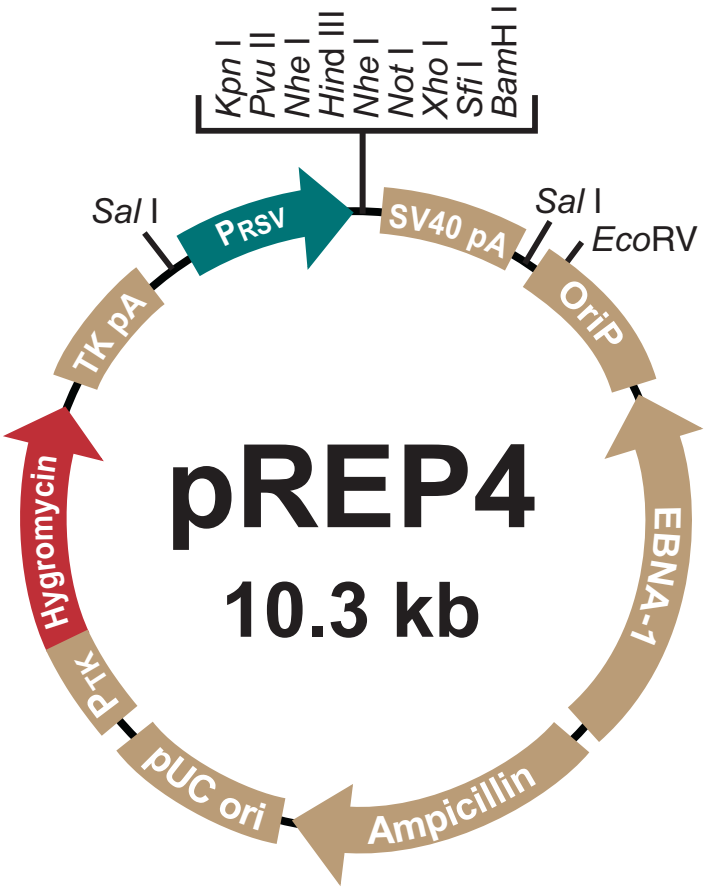
- Always use early-passage cells. Grow and freeze multiple vials of transfected cells to ensure that you have an adequate supply of early-passage cells.
- Always maintain cells in medium containing 50 µg/mL hygromycin.
- Do not maintain cells in continuous culture for longer than 6 months.

Appendix

pREP4 Vector

Map of pREP4

The figure below summarizes the features of the pREP4 vector. The sequence for pREP4 is available at www.invitrogen.com or by contacting Technical Support (see page 12).



Comments for pREP4:
10349 nucleotides

RSV LTR promoter: bases 1-571
Multiple cloning site: bases 575-632
SV40 polyadenylation signal: bases 641-882
OriP: bases 1332-3306
EBNA-1 gene (complementary strand): bases 3607-5532
Ampicillin resistance gene: bases 6158-7018
pUC origin: bases 7027-7802
TK promoter: bases 8524-8686
Hygromycin resistance gene: bases 8750-9892
TK polyadenylation signal: bases 9893-10054

Continued on next page

Features of pREP4

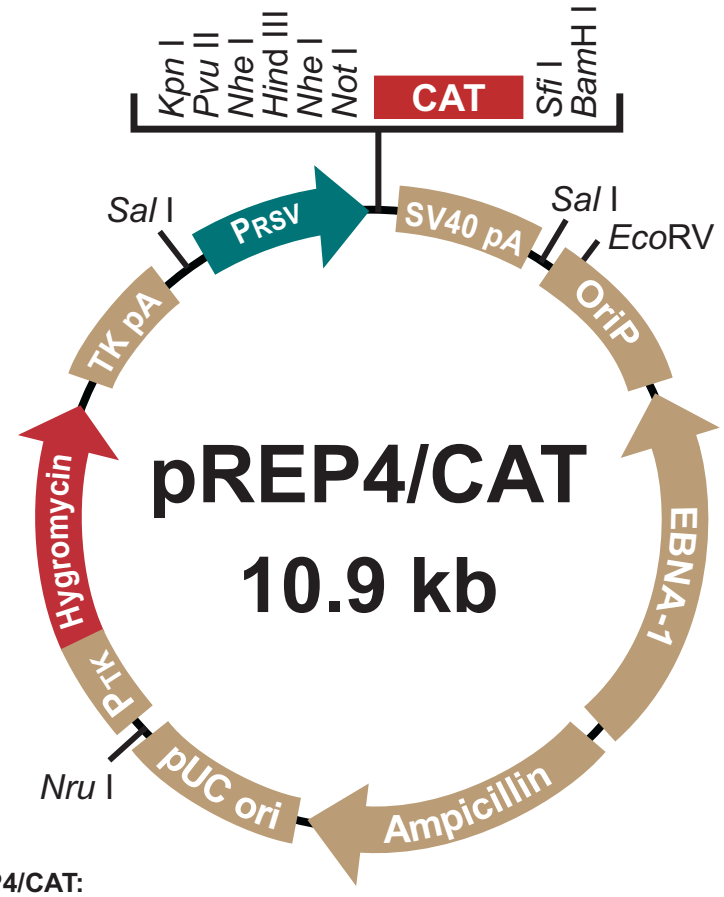
pREP4 (10,349 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Rous Sarcoma Virus long terminal repeat (RSV LTR) enhancer/promoter	Allows for efficient, high-level expression of your recombinant protein.
Multiple cloning site	Allows for insertion of your gene and facilitates cloning.
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA.
EBV origin of replication (oriP) and nuclear antigen (EBNA-1)	High-copy Episomal replication in primate and canine cell lines (Reisman and Sugden, 1986; Yates *et al.*, 1985).
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli.*
pUC origin	High-copy number replication and growth in *E. coli.*
Herpes Simplex Virus thymidine kinase (TK) promoter	Allows for efficient, high-level expression of the hygromycin resistance gene (McKnight, 1980).
Hygromycin resistance gene	Selection of stable transfectants in mammalian cells (Gritz and Davies, 1983; Palmer *et al.*, 1987).
Herpes Simplex Virus thymidine kinase (TK) promoter polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA.

pREP4/CAT

Map of pREP4/CAT

The figure below summarizes the features of the pREP4/CAT vector. The nucleotide sequence for pREP4/CAT is available at www.invitrogen.com or by contacting Technical Support (see page 12).



Comments for pREP4/CAT:
10849 nucleotides

RSV LTR promoter: bases 1-571
Chloramphenicol acetyl transferase (CAT) gene: bases 630-1289
SV40 polyadenylation signal: bases 1345-1586
OriP: bases 2004-3979
EBNA-1 gene (complementary strand): bases 4280-6207
Ampicillin resistance gene: bases 6833-7693
pUC origin: bases 7702-8477
TK promoter: bases 8845-9007
Hygromycin resistance gene: bases 9071-10081
TK polyadenylation signal: bases 10096-10364

Accessory Products

Additional Products

The following additional products may be used with the pREP4 vectors. For more information, visit www.invitrogen.com or contact Technical Support (see page 12).

Item	Quantity	Cat. no.
One Shot® TOP10 Chemically Competent *E. coli*	20 reactions	C4040-03
One Shot® TOP10 Electrocomp™ *E. coli*	20 reactions	C4040-52
Ampicillin	200 mg	11593-027
Carbenicillin	5 g	10177-012
PureLink™ HQ Mini Plasmid Purification Kit	100 preps	K2100-01
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
Lipofectamine™ 2000 Reagent	1.5 mL	11668-019
FAST CAT® Chloramphenicol Acetyltransferase Assay Kit	100 assays	F-2900

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
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For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

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MSDS

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